

Influence of elastin-derived peptides, glucose, LDL and oxLDL on nitric oxide synthase expression in human umbilical artery endothelial cells

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Endothelial dysfunction plays an important role in the development of atherosclerosis. Elastin-derived peptides (EDP), hyperglycemia, hypercholesterolemia and oxidized LDL have a proven proatherosclerotic potential. Nitric oxide generated by endothelial nitric oxide synthase (eNOS; EC 1.14.13.39) is an important vasorelaxant. Here we studied the influence of those proatherosclerotic factors on eNOS gene and protein expression in artery-derived endothelial cells. Human umbilical artery endothelial cells (HUAEC) were incubated with or without: glucose (270 mg/dl), LDL (200 mg/dl), oxidized LDL (oxLDL 25 mg/dl) or κ -elastin (0.5 and 2.5 μ g/ml). Gene expression was assessed by real time RT-PCR, whilst the eNOS protein by ELISA. In cells incubated with 2.5 μ g/ml of κ -elastin, a 31 % increase of eNOS mRNA expression was observed, but the protein level remained unchanged. OxLDL, LDL and glucose decreased the eNOS protein level by 74 %, 37 % and 29 %, respectively. OxLDL decreased eNOS mRNA by 42 %. LDL non-significantly decreased eNOS mRNA expression. An elevated glucose level did not affect the eNOS mRNA expression. Hyperglycemia and an elevated level of LDL, particularly oxLDL, decreased the level of eNOS protein in endothelial cells. As κ -elastin did not decrease the expression of eNOS gene in HUAEC, the proatherogenic properties of elastin-derived peptides are unlikely to be due to their influence on eNOS.

Keywords: endothelium, elastin-derived peptides, EDP, atherosclerosis, hyperglycemia, LDL, oxidized LDL, nitric oxide synthase, eNOS

Received: 05 March, 2011; **revised:** 27 March, 2011; **accepted:** 09 July, 2011; **available on-line:** 11 July, 2011

INTRODUCTION

Human endothelium covers the inner surface of blood vessels and constitutes a huge endocrine gland. The theory of unified response to injury, formulated by Ross (1999), postulates a stereotypic vascular wall reaction in response to various types of endothelial injury. When the endothelium remains undamaged, the vasodilative function prevails. Endothelial damage shifts the balance towards vasoconstriction and atherothrombosis. Nitric oxide generated by nitric oxide synthase (NOS) is a potent vasorelaxant. Although endothelial nitric oxide synthase (eNOS) is a constitutively expressed enzyme, its activity and expression level is influenced by certain factors.

A decrease in nitric oxide bioavailability may be caused by many factors: decreased expression of eNOS, a lack of eNOS substrates or cofactors, accelerated NO degradation or inactivation of eNOS due to dephosphorylation of Ser1177 (Cai & Harrison, 2000). The most important cause of endothelial dysfunction is accelerated NO degradation by reactive oxygen species (ROS), especially superoxide anion $O_2^{\cdot-}$. The reaction between NO and $O_2^{\cdot-}$ is extremely rapid. It occurs three times faster than the dismutation of $O_2^{\cdot-}$ by superoxide dismutase (SOD). The product of this reaction — peroxynitrite — is a powerful trigger of p38 mitogen-activated protein kinase (MAPK) phosphorylation and activation in cells of cardiac, vascular and neural origin (Liaudet *et al.*, 2009). In view of the effect of MAPK in triggering vascular smooth muscle cells' hypertrophy and proliferation, blocking the MAPK cascade probably could reverse vascular dysfunction in patients with heart failure (Ogut & Brozovich, 2008). Even a moderate increase in $O_2^{\cdot-}$ production may cause a serious depletion of NO. This may lead to oxidative stress and endothelial dysfunction. Inactivation of NO by oxidative stress is involved in many conditions, such as atherosclerosis, diabetes, hypertension and hypercholesterolemia. The most important enzymatic systems involved in ROS production are: NADH/NADPH oxidase, xanthine oxidase and uncoupled eNOS. Nitric oxide is a potent activator of soluble guanylyl cyclase (sGC) which produces cyclic guanosine monophosphate (cGMP). cGMP activates protein kinase G (PKG) which leads to calcium-dependent smooth muscle relaxation.

Elastin-derived peptides (EDP) are produced in the process of elastin fiber degradation. EDP occur in human blood in a wide range of concentrations, from 10^{-6} to 10^{-2} mg/ml (Fulop *et al.*, 1990). Oligopeptide sequences VGVAPG are present in both insoluble elastin and EDP. As these sequences activate the elastin receptor, EDP can exert most of elastin's biological effects.

Elastin shows slow turnover, which is accelerated in atherosclerosis, diabetes, lung emphysema, arthritis or neoplasms (Hornebeck & Robert, 1977). Elastin-derived peptides stimulate synthesis and the release of metalloproteases and reactive oxygen species. In experimen-

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Abbreviations: EDP, elastin-derived peptides; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HUAEC, human umbilical artery endothelial cells; LDL, low density lipoprotein; MAPK, mitogen-activated protein kinase; NOS, nitric oxide synthase; oxLDL, oxidized low density lipoprotein; PBS, phosphate-buffered saline

Table 1. Sequences of primers and probes used in quantitative RT-PCR of eNOS and GAPDH genes

GAPDH-Forward	5'-GAA GGT GAA GGT CCG AGT-3'
GAPDH-Reverse	5'-GAA GAT GGT GAT GGG ATT TC-3'
GAPDH-Probe	5'-(FAM)-CAA GCT TCC CGT TCT CAG CC-(TAMRA)-3'
eNOS- Forward	5'-GTG GCT GTC TGC ATG GAC CT-3'
eNOS-Reverse	5'-CCA CGA TGG TGA CTT TGG CT-3'
eNOS-Probe	5'-(FAM)-AGT GGA AAT CAA CGT GGC CGT GCT-(TAMRA)-3'

tal studies, rabbits receiving EDP injections developed atherosclerosis (Gmiński & Drózdź, 1991). Moreover, as children from families with high risk of atherosclerosis present a significantly increased serum level of EDP, it seems that disturbances of elastin metabolism may play a primary role among the risk factors for atherosclerosis (Gmiński *et al.*, 1991). Accordingly, we endeavored to evaluate the mechanism of atherosclerosis induction by EDP and their influence on eNOS expression.

Low density lipoproteins, oxLDL and hyperglycemia are considered potent factors that can negatively affect the arterial wall. Diabetes mellitus causes many vascular complications classified as macro- or microangiopathies. Diabetes mellitus increases up to 4-fold the risk of atherosclerotic complications as compared to individuals without carbohydrate metabolism disturbances. Attempts have been made to evaluate the influence of the above-mentioned factors on the eNOS gene expression, however, the results are divergent (Braam & Verhaar, 2007).

MATERIALS AND METHODS

Endothelial cell culture. Human umbilical artery endothelial cells (HUAEC) were used in this experiment. Umbilical cord was chilled in Hank's solution immediately after delivery. The cells were isolated by mild digestion of the umbilical artery with 0.1% collagenase dissolved in M199 culture medium (Gibco). HUAEC were incubated in EBM-2 culture medium supplemented with 10% foetal bovine serum and EGM-2 MV SingleQuots (Lonza) at 37°C in 5% CO₂ humidified incubator on fibronectin-coated (Sarstedt) tissue culture flasks. The cells were detached from the flasks following 1% trypsin (Gibco) treatment. After each passage, the cells were incubated for 48 h to ensure monolayer. HUAEC were identified using monoclonal antibody against von Willebrand factor and by their typical morphology. Cell viability and morphology were assessed for each experimental group. The procedure of cell isolation and the experimental protocol were approved by the local Bioethics Committee.

Isolation and oxidation of LDL. Fresh plasma from healthy donors was obtained from the local Blood Bank in Katowice. LDL was isolated from fresh human plasma according to Garland's protocol (Garland, 2002). LDL solution was concentrated in Millipore Ultrafree-CL centrifugal ultrafilters. The collected stock solution was dialyzed in dialysis tubes MWCO 50000 (Spectrum Labs) against a PBS buffer with 0.2 mM EDTA, in the dark, at 4°C (5 ml against 1 l of PBS, 3 days, two buffer changes). The part of LDL that was to undergo oxidation was dialyzed against PBS without EDTA. The oxidation of LDL was induced by incubation in dialysis tubes with PBS buffer supplemented with 10 µM Cu²⁺, in the dark, at 21°C for 24 h (Galle & Wanner, 1998).

The oxidized LDL was dialyzed for 3 days against three changes of the PBS buffer with EDTA, in the dark, at 4°C. After the final sterile filtration (Millex, Millipore), LDL and oxLDL were stored refrigerated under argon for no longer than 2 weeks. The concentration of LDL was determined by total cholesterol assay. Additionally, the total LDL-protein concentration was determined by the bicinchoninic acid method (Sigma). LDL and oxLDL purity was checked spectrophotometrically and by electrophoresis (Paragon Lipo, Beckman Coulter).

eNOS protein assay. Immediately after incubation, the cells were detached from flasks by trypsinization, washed twice with PBS buffer and centrifuged (5 min, 2000 rpm). They were then lysed with 0.5 ml of cooled Cell Lysis Solution (R&D). The eNOS protein assay was performed with the use of the human eNOS Immunoassay kit (R&D), in compliance with manufacturer's instructions. Absorbance was measured at 450 and 545 nm with a Stat Fax 2100 Microplate Reader. The total protein concentration was determined with the bicinchoninic acid method.

RNA isolation. Cytoplasmic RNA was isolated using the RNeasy Mini Kit (Qiagen) in compliance with manufacturer's protocol. Approximately 10⁵ cells were lysed with 175 µl of sterile RLN buffer containing: 50 mM Tris/HCl, 140 mM NaCl, 1.5 mM MgCl₂, and 0.5% IGEPAL CA630, pH 8.0. Immediately before isolation, 2 mM dithiothreitol was added to the RLN buffer. The lysis was performed for 5 minutes in an ice bath. The lysate was centrifuged (2 min, 2000 rpm) and the supernatant without nuclei was transferred to another test-tube. The subsequent steps of the procedure were carried out in compliance with the manufacturer's manual. The purity and concentration of the isolated RNA were measured spectrophotometrically using GeneQuant II.

Quantitative RT-PCR. The quantitative measurement of gene expression was performed with an Abi Prism 7000 (Applied Biosystems). The primers and probe for eNOS were based on sequences described previously (Dötsch *et al.*, 2001) and corrected by the author (unpublished). The primers and probe for the housekeeping gene (GAPDH) were synthesized according to the Applied Biosystems recommendations. To avoid potential amplification from DNA, both amplicons spanned introns (Table 1).

For amplification, the commercially available TaqMan EZ RT-PCR Kit (Applied Biosystems) and dNTP (Fermentas) were used. The thermocycler parameters were: 58°C for 32 min (reverse transcription), 95°C for 2 min (initial denaturation), followed by 35 cycles at 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 68°C for 30 s (extension). The expression of endothelial nitric oxide synthase was determined by means of the comparative $\Delta\Delta\text{CT}$ method. Before its application for quantitation, a validation experiment was performed. A serial dilution of RNA was amplified under the same conditions to demonstrate that the efficiency of target (eNOS) and reference (GAPDH) amplifications were approximately equal.

Experiment design. In all experiments, the fourth passage of cells was used. HUAEC were incubated with the tested substances for 48 h. Before the experiment, cells were incubated for 24 h in serum-free medium. HUAEC from each flask were halved. The isolation of

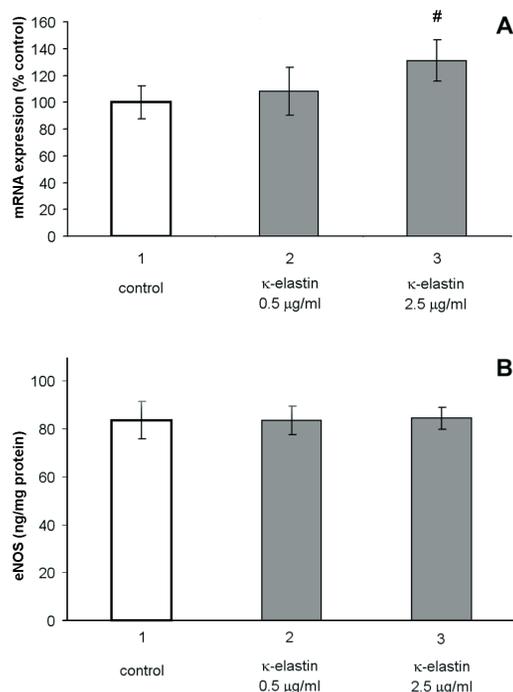


Figure 1. Effects of κ -elasticin on eNOS mRNA and protein quantity in HUAEC

Cells were incubated with or without (control) κ -elasticin (0.5 or 2.5 μ g/ml) for 48 hours. eNOS mRNA (A) or protein (B) was quantitated as described in Materials and Methods. The results represent mean \pm S.D. of 4 experiments. [#] $p \leq 0.01$ vs. control.

RNA and total protein was performed for each part of the cells as described previously.

Statistical analysis. All values are expressed as mean \pm S.D. To determine the significance of differences between groups, the ANOVA test followed by Tukey's and Scheffe's *post hoc* tests were used. To compare the study groups exclusively with the control, the Dunnett's test was used.

RESULTS

κ -Elasticin at a concentration of 0.5 μ g/ml did not affect eNOS mRNA expression or the protein level. In cells incubated with 2.5 μ g/ml of κ -elasticin, a 31% ($p \leq 0.001$) increase in eNOS mRNA expression was observed, whilst the protein quantity remained unchanged (Fig. 1). OxLDL decreased the eNOS mRNA level by 42% ($p \leq 0.001$). Although LDL caused a decrease in eNOS expression, the effect was not statistically significant. An elevated glucose concentration did not affect mRNA expression of eNOS (Fig. 2).

OxLDL, LDL and elevated glucose decreased eNOS protein concentration by 74%, 37% and 29% (all $p \leq 0.001$), respectively.

Cells stimulated with high glucose and κ -elasticin showed a 24% ($p \leq 0.001$) increase in the amount of eNOS protein, compared to the cells stimulated by glucose only. In the remaining groups tested, the addition of κ -elasticin to LDL or oxLDL did not affect eNOS protein or mRNA concentrations (Fig. 3).

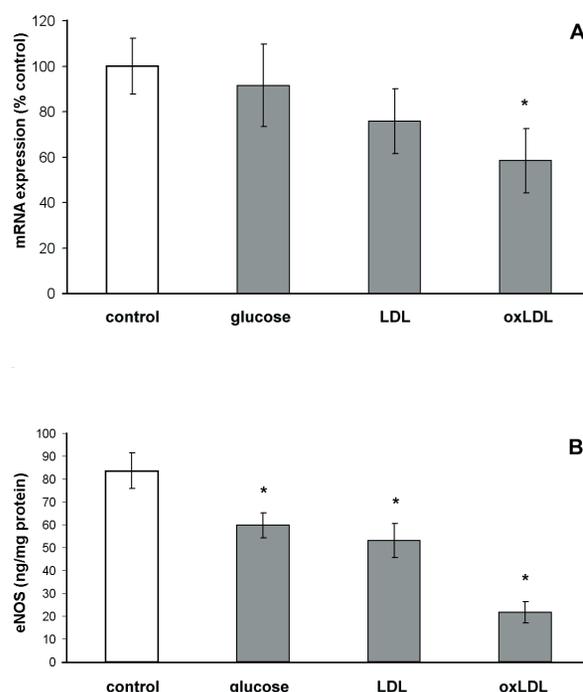


Figure 2. Effects of glucose, LDL, and oxLDL on eNOS mRNA and protein quantity in HUAEC

Cells were incubated with or without (control) glucose (270 mg/dl), LDL (200 mg/dl) and oxLDL (25 mg/dl) for 48 hours. eNOS mRNA (A) or protein (B) was quantitated as described in Materials and Methods. The results represent mean \pm S.D. of 4 experiments. ^{*} $p \leq 0.001$ vs. control.

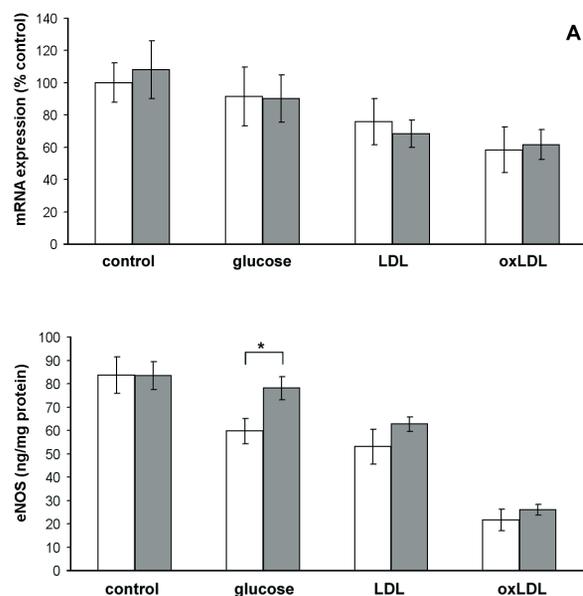


Figure 3. Comparison of the effect of glucose, LDL and oxLDL on eNOS mRNA and protein quantity in the absence (white bars) or presence (gray bars) of κ -elasticin in HUAEC

Cells were incubated with glucose (270 mg/dl), LDL (200 mg/dl), oxLDL (25 mg/dl) and κ -elasticin (0.5 μ g/ml) for 48 hours. eNOS mRNA (A) or protein (B) was quantitated as described in Materials and Methods. The results represent mean \pm S.D. of 4 experiments. ^{*} $p \leq 0.001$ presence vs. absence of κ -elasticin.

DISCUSSION

Although several studies evaluating the influence of various proatherosclerotic factors on the eNOS gene expression have been performed, their results are divergent. It is hypothesised that the physiology of human umbilical arterial endothelial cells (HUAEC) may differ from human umbilical vein endothelial cells (HUVEC) used in the majority of studies. Because of the similarity between the various types of human arterial endothelial cells, in our study HUAEC were used. The LDL concentration chosen for the presented study can be observed in humans with dyslipidemia and other metabolic disorders. It is a concentration that leads to atherosclerosis *in vivo* and is considered as "very high" according to the National Cholesterol Education Program Adult Treatment Panel III (Stone *et al.*, 2005). Our experiment proved that LDL cholesterol at 200 mg/dl significantly decreased the amount of eNOS protein in the studied endothelial cells. The 24% reduction of eNOS gene expression was not statistically significant. Pritchard *et al.* (2002) observed an increase in the amount of eNOS protein caused by LDL in bovine arterial endothelial cells (BAEC). Opposite results were reported by Jiménez *et al.* (2001) who compared endothelial cells from the aortas of rabbits on hypercholesterolemic and standard diet. The reduction of eNOS was observed both on the level of gene expression and the protein amount, but they achieved a very high plasma cholesterol concentration (900 mg/dl). The reduction of the amount of eNOS in human endothelial cells observed in our study may be caused by decreased eNOS mRNA stability.

There is strong evidence regarding the influence of oxidized LDL on endothelial nitric oxide synthase. Liao *et al.* (1995) reported that the expression of eNOS mRNA in cultured endothelial cells was decreased by oxLDL at 15–60 mg/dl in a proportional manner. In the presented study, oxLDL concentration of 25 mg/dl was chosen and it did not present cytotoxic properties. Moreover, oxLDL had the strongest inhibitory effect on both the eNOS gene expression and protein quantity.

The results of studies evaluating the influence of hyperglycemia on eNOS gene expression are inconsistent, probably due to the diversity of experimental models used. Cosentino *et al.* (1997) evaluated eNOS gene expression in cultured endothelial cells derived from the human aorta. Five days of incubation with the addition of 400 mg/dl glucose resulted in a 2-fold increase of both mRNA and eNOS protein. Srinivasan *et al.* (2004) incubated aortic endothelial cells with the addition of 450 mg/dl glucose. Initially, an increase in both eNOS mRNA and protein amount were observed, however, after the first 24 hours the amount of eNOS protein began to decrease, whilst mRNA remained elevated. Our experiment was performed with a lower glucose concentration (270 mg/dl), which was closer to the blood glucose level observed in patients with uncontrolled diabetes mellitus. After 48 hours of incubation, a 29% decrease of eNOS protein level was observed ($p \leq 0.001$), whilst the gene expression remained unchanged.

As α -elastin displays all the biological effects of EDP and is widely used in experiments, it has been adopted as a HUAEC-stimulating factor. The α -elastin concentrations used in our experiments were chosen according to Faury *et al.* (1995) report. In an earlier study they demonstrated that α -elastin in the concentration range between 0.1 ng/ml and 1 μ g/ml added to rat aortic ring *ex vivo*, precontracted with noradrenalin, induced

an endothelium-dependent vasodilatation. This effect was attenuated by an eNOS inhibitor (L-NAME) and a cyclooxygenase inhibitor (indomethacin), thereby suggesting a number of mechanisms of EDP action. They also stimulated human umbilical vein endothelial cells (HUVEC) with 1 μ g/ml of α -elastin. In this conditions, α -elastin induced 1.73-fold increase of intracellular Ca^{2+} (Faury *et al.*, 1998). α -Elastin was used in our experiment at 0.5 μ g/ml and 2.5 μ g/ml. These concentrations were chosen to elucidate the effects on eNOS expression at the mRNA and protein levels. Both in humans and experimental animals, EDP increased the elastolytic activity relating to the endothelium, smooth muscle cells and inflammation site infiltrating cells. An augmented elastolytic activity leads to an accelerated elastin degradation and eventually causes a release of huge amounts of EDP. This phenomenon is referred to as "a self-propelled vicious circle" of stimulations (Robert *et al.*, 2010). Even 1 μ g/ml of α -elastin accelerates neutrophil-induced LDL oxidation *in vitro* (Fulop *et al.*, 2005). Moreover, it has been shown that α -elastin at 0.5 μ g/ml and 2.5 μ g/ml reflects both the EDP concentrations observed in people and concentrations leading to aorta dilatation *ex vivo*.

So far, there has been no research on the influence of elastin-derived peptides focusing simultaneously on eNOS gene expression and protein level. In our experiment, neither low (0.5 μ g/ml) nor high (2.5 μ g/ml) α -elastin concentrations influenced eNOS protein expression. Only the high α -elastin concentration resulted in a 31% increase of eNOS mRNA expression ($p \leq 0.01$). Analyzing the influence of an elevated glucose concentration with and without the addition of α -elastin to the culture medium, we observed that α -elastin led to an increase in the quantity of eNOS protein. In the analogous experiment with LDL, a similar effect was obtained, although it did not reach the level of statistical significance. Elastin-derived peptides present proatherosclerotic properties and their pathogenic activity needs not to be related to the influence on eNOS gene expression. The blood concentration of EDP increases with age. Taking into consideration that the society is getting older, further research on the influence of EDP on the development of atherosclerotic process is crucial.

Conflict of interest statement

The authors state that there are no conflicts of interest.

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